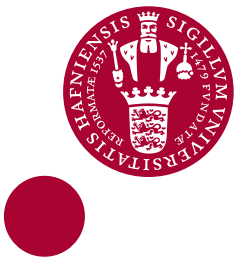

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Nano-nutrition of chicken embryos. The effect of silver nanoparticles and glutamine on molecular responses, and the morphology of pectoral muscle.

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Abstract

Background: It has been demonstrated that concentrations of certain amino acids in the egg, in late-term embryos, are not sufficient to fully support embryonic development. One of the methods to assure an adequate nutrient content in the egg is *in ovo* administration of nutrients, which increases hatching weight and the size of the breast muscle. The small size of silver nanoparticles allows for penetration inside tissues and even enables them to cross cell membranes. Indeed it has been shown that nanoparticles of silver applied *in ovo* can up-regulate the expression of fibroblast growth factor and vascular endothelial growth factor. We have therefore tested if silver nanoparticles can affect muscle development of chicken embryos and, furthermore, if they can be used in *in ovo* nutrition as carriers of nutrients e.g. glutamine into muscle cells.

Methods: 160 broiler eggs were randomly divided into the control group (Control) without injection and injected groups with hydrocolloids of nanoparticles of silver (Nano-Ag), glutamine (Glu) and the complex of nanoparticles of silver and glutamine (Nano-Ag/Glu). The embryos were evaluated on day 20 of incubation. Samples of the breast muscles were collected for gene expression analysis or fixed for electron microscopy preparation.

Results: Results indicate a significant role of silver nanoparticles and the complex Nano-Ag/Glu in muscle development.

Conclusion: Nanoparticles of Ag, glutamine and the complexes of Ag and glutamine were without negative effects on embryo development. Nanoparticles of Ag and the complex of Ag with glutamine increased the number of nuclei per cell number and also fiber area. Furthermore, the complex of Ag with glutamine increased muscle mass.

Keywords: silver nanoparticles, muscle, glutamine, nutrition, gene expression, chicken embryo.

Introduction

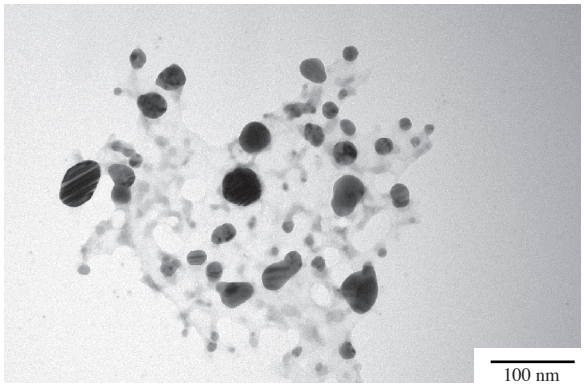
Muscle growth and development consist of two periods, hyperplasia and hypertrophy[1]; while hyperplasia (an increase in the number of muscle cells) mostly occurs during embryogenesis the hypertrophy (an increase in the volume of muscle cells) takes place during the post hatching growth[2,3]. During the embryonic period of muscle development there are three distinct stages: the formation of myoblasts,

the fusion of myoblasts to form myotubes and the conversion of myotubes to form myofibers[4]. This pattern of development is regulated and controlled by a variety of genes and depends on the availability of nutrients stored within eggs. Consequently, an adequate supply of nutrients is critical for embryonic development. One of the methods to assure an adequate nutrient content in the egg is *in ovo* administration of nutrients, which increases hatching weight and the size of breast muscle[5, 6, 7, 8]. However, this method has drawbacks, including interference with embryo homeostasis and the risk of microbial hazards not to mention the proper transport and distribution of supplemented nutrients.

Silver nanoparticles have different physical and chemical characteristics compared to their larger equivalents because of a very high surface to volume ratio, physical activity and chemical stability[9, 10]. The small size of nanoparticles allows for penetration inside tissues and even enables them to cross cell membranes[11, 12, 13]. *In vivo* studies demonstrated that silver nanoparticles used at a low level were non-toxic and did not affect immune responses[14, 15].

Recently, it has been shown that nanoparticles of silver applied *in ovo* can up-regulate the expression of *fibroblast growth factor 2* (*FGF2*) and *vascular endothelial growth factor* (*VEGF*)[11]. *FGF2* stimulates satellite cell proliferation and differentiation, as well as angiogenesis[1], while *VEGF* stimulates vasculogenesis and angiogenesis of muscle tissue[16]. Therefore, it is important to investigate whether silver nanoparticles can affect the expression of the other genes involved in chicken embryo muscle development. The key genes responsible for sustained muscle growth and development during embryogenesis are *myogenic differentiation factor* (*MyoD1*), *pair box transcription factor* 7 (*Pax 7*), *proliferating cell nuclear antigen* (*PCNA*) and *ATPase* (*ATP1A1*). MyoD1 belongs to the family of 4 basic helix-loop-helix transcription factors, which activate the myogenic programme and control muscle-specific gene expression[17], whilst *PCNA* plays a key role in embryonic DNA synthesis during the cell cycle within the nuclei. Skeletal muscle growth is also related to the satellite cell population, which is controlled by *Pax 7*. *ATP1A1* is involved in maintaining electrochemical gradients of Na^+ and K^+ ions across the plasma membrane, which is responsible for osmoregulation, electrical excitability, and consequently for energy metabolism of the muscle cells.

Figure 1.



Transmission electron microscopy image of silver nanoparticles manufactured by the electric non-explosive method.

It has been demonstrated that concentrations of certain amino acids in the egg, in late-term embryos, are not sufficient to fully support embryonic development[18]. During embryogenesis, energy and nutrients are mainly provided from the yolk, which is rich in lipids but low in carbohydrates[8]. Consequently, the embryo, and also post hatched chicken, depend on gluconeogenesis from amino acids[19], which in turn decreases protein deposition in muscles, affecting the development of muscles[5, 20]. Moreover, after hatching when energy reserves are low, amino acids from pectoral muscle form the main substrates for gluconeogenesis[19]. Therefore, delivering extra amino acids to embryos could reduce such protein catabolism in muscles.

Glutamine is an important substrate for the synthesis of amino acids necessary for muscle development; it is involved in gluconeogenesis and in maintaining the protein – energy balance in the muscle cells[18, 21]. Furthermore, glutamine is not only essential for protein synthesis, it is also a key source of nitrogen for the synthesis of purines, and it forms a source of carbon for energy production[23].

It has been demonstrated that nanoparticles of gold can affect muscle development and act as carries of nutrients into muscle cells[21]. However, only a few results indicate the potential effects of silver on chicken development[23, 24] and to date there is no information available concerning the application of silver nanoparticles as carries of nutrients into embryo tissues. In this investigation, we hypothesized that silver nanoparticles can affect muscle development of chicken embryos and, furthermore, they can be used in *in ovo* nutrition as carriers of nutrients into muscle cells. Nanoparticles can bypass conventional physiological ways of nutrient distribution and transport across tissue and cell membranes, as well as protect compounds against destruction prior to reaching their targets. In which case *in ovo* administration of nanoparticles, acting as bioactive agents and as carries of nutrients may be seen as a new method of nano-nutrition, providing embryos with bioactive compounds and/or with an additional quantity of nutrients or energy.

The objective of the investigation was to evaluate the effect of nanoparticles of silver, glutamine and complexes of glutamine conjugated with silver nanoparticles on the expression of genes related to embryonic muscle development (*FGF2, VEGF, MyoD1, PAX7, PCNA*, and *ATP1A1*) and on the morphological characteristics of muscle.

Material and methods

Experimental design

Ross × Ross 308 (broiler line) chicken eggs were obtained from a commercial hatchery. 160 eggs were randomly divided into four groups (4 x 40 eggs): without injection (Control), injected with hydrocolloid of Ag nanoparticles (Nano-Ag), injected with hydrocolloid of glutamine (Glu), injected with hydrocolloid of Ag nanoparticles with glutamine (Nano-Ag/Glu). Eggs were stored in a refrigerator (10 °C) for 1–3 days before being placed in an incubator. At day 1 of incubation,

the eggs were numbered, weighed (60.00 ± 1.36 g) and injected according to the above treatment descriptions. The eggs were injected into the air sac with 0.3 ml of solutions using a sterile 27 gauge, 20 mm needle. Immediately after the injection, the hole was sealed with sterile tape and the eggs were replaced into an incubator. The eggs were incubated for 20 days under standard conditions (temperature 37.8 °C, humidity 55%, turned once per hour during the first 18 days, at a temperature of 37 °C and humidity 60% from day 19).

The embryos were evaluated on day 20 of incubation. The development status of chicken embryos was compared with the standard described by Hamburger and Hamilton (1951), furthermore, embryos were examined for any genetic defects[25]. The embryos were weighed together with the yolk sack, decapitated, and blood samples from the carotid artery were taken and collected in Eppendorf tubes (Eppendorf AG, Hamburg, Germany). The samples were incubated at room temperature until clotting and then centrifuged for 10 minutes (14.500 x g). The resulting blood serum samples were stored at -30°C until further analysis. The liver, heart, spleen and breast muscle were collected and weighed. Samples of the breast muscles were collected in RNAlater® ribonucleic acid (RNA) stabilization solution (Applied Biosystems/Ambion, Austin, TX, USA) for gene expression analysis or fixed in glutaraldehyde for electron microscopy preparation.

>> Nano-particles of silver affect muscle morphology

Solutions

Nanoparticles

The hydrocolloid of Ag-Nano (Figure 1) was obtained from Nano-Tech (Warsaw, Poland) and was produced by a non-explosive high voltage patented method (Polish Patent 3883399) from high purity metals (99.9999%) and high purity demineralized water. The concentration of nanoparticles in the hydrocolloids was 50 ppm and the particle size ranged from 2 to 35 nm for Nano-Ag based on the transmission electron microscope (TEM) evaluation as previously described[26].

Glutamine solutions

Pure L-Glutamine (Merck, Darmstadt, Germany) was dissolved in ultra-pure water at a concentration of 25 mg/ml. Furthermore, a hydrocolloid of Ag nanoparticles conjugated with glutamine was prepared by the self-organisation process using sonification for 30 min at 30°C in an ultrasonic bath. The concentration of glutamine in the solution was 25 mg/ml.

Oxygen consumption

The oxygen (O_2) consumption was measured at days 10, 13, 16 and 19 of incubation, as previously described [27]. The eggs were weighed and candled prior to the measurements to check for the presence of embryos. Eggs without an embryo were discarded and replaced with eggs of the same age from the same treatment kept in the incubator as reserves. The consumption of O_2 was measured

according to the paramagnetic principle in an open-air-circuit respiration unit (Micro-Oxymax calorimeter from Columbus Instruments, Columbus, Ohio, USA), equipped with four respiration chambers with a volume of 2000 cm^3 each. The temperature and relative humidity were maintained similar to that of the incubator. Six eggs from each treatment were placed in the respiration chambers and measured for 3 h from 9:00 to 12:00, followed by another six eggs from the same treatment measured from 13:00 to 16:00. After each measurement, the eggs were put back into the incubator. The measurements were standardized to a 50 g egg mass in order to account for differences in weight during each measurement.

Health and metabolic indices in the blood serum of chickens

The concentration of magnesium, calcium, phosphorus, triglycerides, cholesterol in very-low-density lipoprotein, glucose, and the activity of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and lactate dehydrogenase in blood serum was measured by dry chemistry methods (Vitros DT 60 II, Johnson and Johnson, New Brunswick, USA).

Microscopy visualization

For observations using the TEM, tissues, obtained immediately after dissection, were cut into pieces of about 1 mm^3 and fixed (60 min) in a 3% glutaraldehyde solution (Merck, Darmstadt, Germany) in 0.1 M sodium phosphate buffer (pH 7). Samples were then rinsed (2 x 5 min) in the same buffer and transferred to a 1% osmium tetroxide solution (Electron Microscope Science, Sigma-Aldrich) in 0.1 M phosphate buffer (pH 6.9) for 1 hour. Subsequently, the samples were rinsed in 0.1 M sodium phosphate buffer (5 min), dehydrated in an ethanol (Merck, Darmstadt, Germany) gradient (50-99 %), and impregnated with Epon embedding resin (Merck, Darmstadt, Germany). The next day, the samples were embedded in the same resin and baked for 48 hours at 60°C. The blocks were cut into semi-thin sections for light microscopy (LM) using an ultra-microtome (Leica, ultracut UCT) and stained with 1% basic Toluidine blue. From selected regions of the tissue, ultrathin sections (50–80 nm) were cut and transferred onto copper grids (200 mesh). Subsequently, the sections were contrasted using 2 % uranyl acetate dihydrate and lead citrate (Reynolds). The structure of the chest muscles was visualized by a JEM-1220 TEM at 80 KeV (JEOL, Tokyo, Japan), with a Morada 11 megapixel camera (Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Morphometry of the breast muscle of chicken embryos (fibre area, cell number per 1200 μm^2
and number of nuclei per 1200 μm^2) was counted using ´´ImageJ´´ software (http://rsb.info.nih.gov/ij/).

Gene expression at the RNA level

The tissue dissected from the breast muscle was homogenized in TRIzol®

Reagent (Life Technologies), and total RNA was extracted according to the manufacturer's instructions. The RNA samples were purified, using the SV Total RNA Isolation System (Promega Corporation, Madison, WI, USA) and quantified using a NanoDrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Using reverse transcriptase with oligo (dT) (Promega) and random primers (TAG Copenhagen A/S, Copenhagen, Denmark), 2 mg of total RNA was reverse transcribed, after which real-time PCR was performed with complementary DNA and gene specific primer pairs (TAG Copenhagen, Denmark) mixed with LightCycler®480 SYBR Green I Master mix (Roche Applied Science, Penzberg, Germany) in a LightCycler®480 real-time PCR system (Roche Applied Science, Penzberg, Germany). The samples were first denatured for 5 minutes at 95°C and then amplified using 45 cycles of 10 seconds at 95°C (denaturation), 10 seconds at 60°C–62°C (annealing), and 9 seconds at 72°C (elongation), followed by quantification. A melting curve program (56°C–97°C with a heating rate of 0.11°C/second and five acquisitions per 1°C) was applied to verify the specificity of the product. For each complementary DNA, the reaction was performed in triplicate. For analyses, relative quantification was applied with *beta-actin* (*ACTB*) used as the housekeeping genes.

>> Nano-particles of silver delivered together with glutamine affect the expression of genes responsible for muscle development during embryogenesis

Gene expression of FGF2 (Fibroblast Growth Factor 2) and VEGF (Vascular Endothelial Growth Factor) at the protein level (ELISA method)

Frozen breast muscle tissue samples were homogenized on ice using RIPA lysis and extraction buffer (Thermo Fisher Scientific) and a Polytron® PT 2100 homogenizer (Kinematica AG, Lucerne, Switzerland). Homogenates were left on ice for 30 minutes and were subsequently centrifuged for 20 minutes (4°C, 12,500 rpm). The supernatant was collected in chilled Eppendorf PCR tubes (Eppendorf AG, Hamburg, Germany). Supernatant samples were divided into two equal portions. One was used to evaluate the total protein concentration (Total Protein Kit, Micro Lowry, Peterson's Modification; Sigma-Aldrich). The second portion was used to perform the enzyme-linked immunosorbent test, using an Enzymelinked Immunosorbent Assay Kit (USCN Life Science, Tokyo, Japan). Reagents and plates were prepared accordingly to the manufacturer's standard procedure and incubated for 25 minutes under standard conditions. The degree of absorption was measured in a microplate reader Infinite®M200 PRO (Tecan Deutschland GmbH, Crailsheim, Germany).

2.8. Statistical methods

Data analysis was carried out using the one-way analysis of variance (ANOVA) test-generalized linear model (Tukey-Cramer) (GLM) procedure in SAS 9.2 (SAS Windows, 2002-2008, version 9.2, SAS Institute INC., Cary, NY, USA). Mean values differing with a $P<0.05$ were considered significantly different.

3. Results

Nano-Ag, Glu and Nano-Ag/Glu did not affect the weight of the liver, heart and spleen of embryos. However, the muscle percentage of body weight was significantly higher with Nano-Ag/Glu treatment compared to the other groups (Table 1). Embryo visualization did not show any genetic defects among the groups. Furthermore, comparison with Hamburger-Hamilton normal stages of chicken embryo development showed that all embryos had developed normally. In addition, all of the biochemical indices measured in the blood serum of chicken embryos did not show significant effects of treatment (Table 2).

Table 1. Average weight of chicken embryos, organs and pectoral muscle

	Treatment								
	Control		Nano-Ag		Glu		Nano-Ag/Glu		
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	P-value
Embryo, % e.w1	78	1.52	76	1.72	76	0.5	75	0.88	0.28
Liver, % b.w2	1.5	0.05	1.4	0.05	1.4	0.04	1.4	0.04	0.095
Heart, % b.w2	0.5	0.01	0.5	0.01	0.5	0.01	0.5	0.01	0.14
Spleen, % b.w2	0.04	0.002	0.05	0.009	0.03	0.002	0.03	0.002	0.07
Muscle, % b.w2	0.81a	0.035	0.80a	0.051	0.72a	0.062	1.04b	0.080	0.003

Within rows: means with different superscript differ significantly ($P < 0.05$).
Abbreviations: 1% of egg weight; 2% of body weight. SEM – standard error of mean. Groups: Control, non injected; Nano-Ag, injected with silver nanoparticles; Glu, injected with glutamine; Nano-Ag/Glu, injected with silver nanoparticles and glutamine.

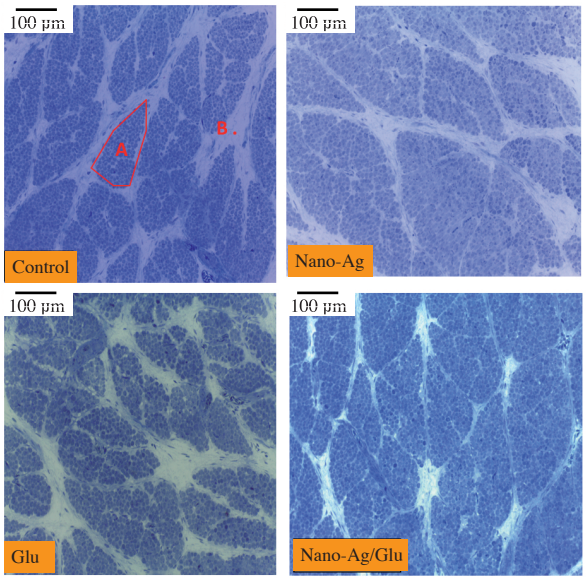
Table 2. Table 2 Biochemical indices measured in the blood serum of chicken embryos

	Treatment								
	Control		Nano-Ag		Glu		Nano-Ag/Glu		
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	P-value
Alkaline phosphatase (U/L)	6125	521	6439	452	7023	807	6945	502	0.68
Aspartate aminotransferase (U/L)	178	21	166	24	126	23	166	27	0.22
Alanine aminotransferase (U/L)	3.7	0.33	4.3	0.33	2.0	0	3.33	0.33	0.06
Lactate dehydrogenase (U/L)	1778	358	2219	709	1360	48.5	2012	122	0.51
Magnesium (mmol/L)	0.9	0.02	0.9	0.01	0.9	0.04	0.9	0.01	0.33
Calcium (mmol/L)	2.3	0.08	2.1	0.12	2.1	0.11	2.1	0.07	0.75
Phosphate (mmol/L)	1.3	0.01	1.5	0.15	1.4	0.03	1.4	0.13	0.64
Triglycerides (mmol/L)	1.0	0.04	1.4	0.11	1.0	0.05	1.0	0.12	0.01
Cholesterol (mmol/L)	11	1.7	12	1.45	11	0.27	10	0.71	0.76
Glucose (mmol/L)	14.9	0.28	14.8	1.02	13.5	0.44	13.3	0.55	0.25

Abbreviations: Groups: SEM – standard error of mean. Groups: Control, non injected; Nano-Ag, injected with silver nanoparticles; Glu, injected with glutamine; Nano-Ag/Glu, injected with silver nanoparticles and glutamine.

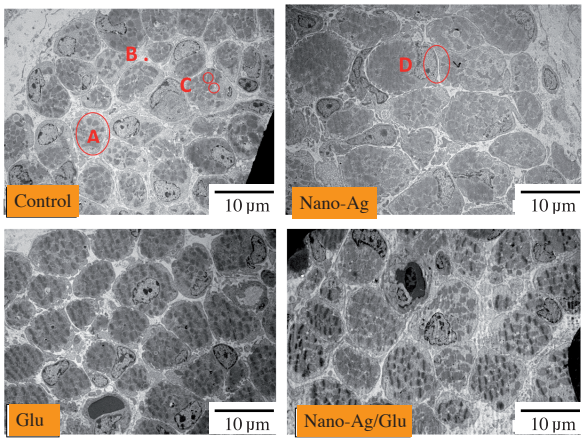
Chicken embryo breast muscle tissues were visualized using LM and TEM, which enabled observation of the structure of the pectoral muscle. There were visual changes in the structure of the cross section of breast muscle in the treated groups compared to the embryos from the control group. According to LM in the Nano-Ag/Glu group, bundles of muscle fibres formed the most compact and rounded structure. In addition, the perimysium in the Nano-Ag/Glu group was smaller compared to the other groups. Furthermore, the visual bundle area was larger in the Nano-Ag and Nano-Ag/Glu groups when compared with the control group (Figure 2).

Figure 2.



Light microscopy image of pectoral muscle (cross section). Groups: Control, non injected; Nano-Ag, injected with silver nanoparticles; Glu, injected with glutamine; Nano-Ag/Glu, injected with silver nanoparticles and glutamine. (A) Fascicle and (B) perymisium.

Figure 3.



Transmission electron microscopy image of pectoral muscle (cross section). Groups: Control, non injected; Nano-Ag, injected with silver nanoparticles; Glu, injected with glutamine; Nano-Ag/Glu, injected with silver nanoparticles and glutamine. (A) Single muscle fibre, (B) endomysium, (C) myofibrils. (D) nucleus.

Visualization of transverse sections of the pectoral muscle samples using TEM showed (Figure 3) that muscles cells from the control group were the smallest and that the space between the cells was wider than in the other groups. Within cells, myofibrils were loosely placed with relatively long distances between myofibrils, moreover, myomere decay, unification of myosin and actin filaments and occasional myelin structures were noticed. The muscle fibre area was larger in Nano-Ag and Nano-Ag/Glu. Furthermore, myofibrils of treated embryos were more concentrated, compact and

Table 3. Morphometry of the breast muscle of chicken embryos

	Treatment								
	Control		Nano-Ag		Glu		Nano-Ag/Glu		
mean	SEM	mean	SEM	mean	SEM	mean	SEM		P-value
Cell number/1200µm2	17.0a	0.27	12.2c	0.25	13.6b	0.58	9.8c	0.31	0.0001
Number of nuclei/1200µm2	7.2	0.67	8.2	0.53	7.8	0.68	6.5	0.33	0.001
Nuclei/Cell	0.42		0.67		0.57		0.67		
Fiber area (µm2)	38a	1.62	79c	3.06	58b	1.87	74c	1.98	0.001

Within rows: means with different superscript differ significantly ($P < 0.05$).
Abbreviations: SEM – standard error of mean. Groups: Control, non injected; Nano-Ag, injected with silver nanoparticles; Glu, injected with glutamine; Nano-Ag/Glu, injected with silver nanoparticles and glutamine.

Figure 4. Day of incubation

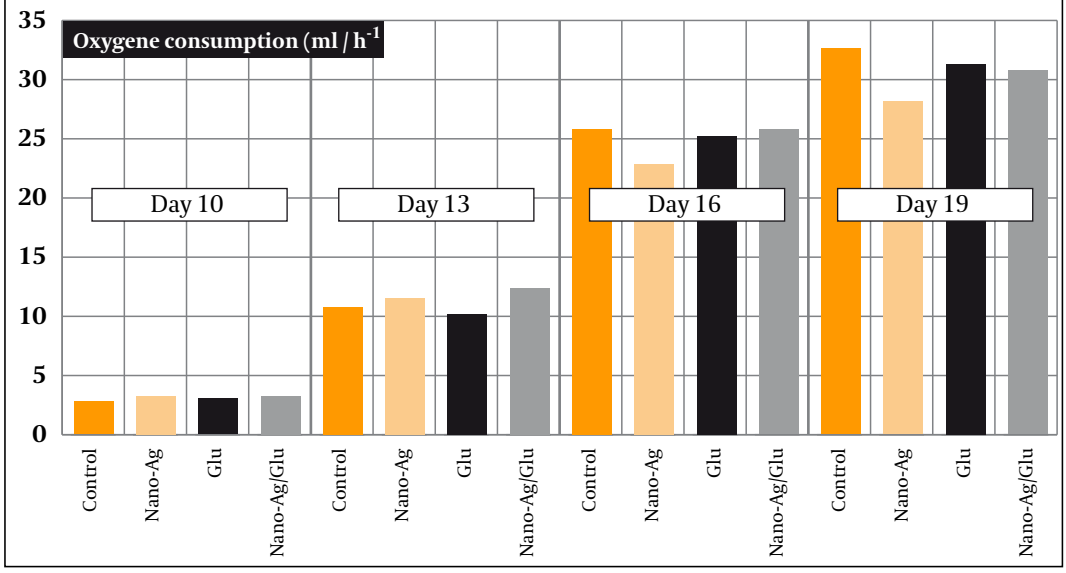


Figure 4. Mean oxygen consumption (ml/h-1) of chicken embryos measured at days 10, 13, 16 and 19 of incubation. Groups: Control, non injected; Nano-Ag, injected with silver nanoparticles; Glu, injected with glutamine; Nano-Ag/Glu, injected with silver nanoparticles and glutamine.

placed more regularly than the controls. Muscle cells of chickens from the Nano-Ag/Glu group were surrounded with regular, smooth and well convex membranes.

Quantitative measurements demonstrated that the control group had a significantly higher number of muscle cells compared to the remaining groups (Table 3). However, significantly the biggest pectoral muscle fibre areas were found in the Nano-Ag (79 μm^2) and Nano-Ag/Glu (74 μm^2) groups. The numbers of nuclei on the quadratic surface of the cross section image (1200 μm^2) were the same for all groups. Although, the proportion between the number of nuclei and the cell number was higher in the Nano-Ag (0.67) and Nano-Ag/Glu (0.67) groups compared to the Glu (0.57) and Control (0.42) groups.

Oxygen consumption (Figure 4) measured at day 10, 13, 16 and 19 of incubation was not significantly different between the treatments. However, at the end of incubation there was a visible decrease in O_2 consumption for the Nano-Ag group compared with the other groups.

At the protein level (Table 4), expression of FGF2 increased significantly for Nano-Ag (46.7) and Nano-Ag/Glu complex (44.4) compared to the control

Table 4 . Gene expression at the protein level (Enzyme-linked immunosorbent assay) in the breast muscle tissue of chicken embryo

	Treatment								
	Control		Nano-Ag		Glu		Nano-Ag/Glu		
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	P-value
FGF	24.4^{a}	3.31	46.7^{b}	1.63	34.7^{ab}	3.82	44.4^{b}	3.21	0.0011
VEGF	32.4^{a}	1.51	62.6^{b}	3.25	46.2^{c}	1.44	40.8^{ac}	2.71	0.0001

Within rows: means with different superscript differed significantly ($P < 0.05$).
Abbreviations: SEM – standard error of mean. Groups: Control, non injected; Nano-Ag, injected with silver nanoparticles; Glu, injected with glutamine; Nano-Ag/Glu, injected with silver nanoparticles and glutamine. FGF2, Fibroblast Growth Factor 2; VEGF, Vascular Endothelial Growth Factor.

group (24.4). There was no significant effect of glutamine (34.7), however, there was a noticeable increase compared to the control group. Furthermore, Nano-Ag (62.6), Glu (46.2) and Nano-Ag/Glu (40.8) significantly increased the expression of VEGF at the protein level compared to that of the Controls (32.4). At the mRNA level (Table 5), the expression of *FGF2* was increased in the Nano-Ag group (0.94) compared to the control group (0.73). Regarding

Table 5. Gene expression at the mRNA level (Polymerase Chain Reaction) in the breast muscle tissue of chicken embryos

	Treatment								
	Control		Nano-Ag		Glu		Nano-Ag/Glu		
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	P-value
FGF2	0.73^{a}	0.031	0.94^{b}	0.047	0.79^{ab}	0.027	0.84^{ab}	0.061	0.008
VEGF	0.57^{a}	0.067	1.34^{b}	0.213	2.08^{c}	0.143	0.78^{a}	0.052	0.0001
PAX7	1.15	0.051	0.91	0.062	1.25	0.218	1.38	0.049	0.087
PCNA	0.92^{a}	0.052	1.01^{a}	0.048	1.07^{a}	0.092	1.54^{b}	0.099	0.001
MyoD1	0.68^{a}	0.033	1.57^{b}	0.141	0.62^{a}	0.048	1.00^{c}	0.074	0.001
ATP1A1	0.92^{a}	0.059	1.38^{b}	0.126	1.13^{ab}	0.126	0.80^{a}	0.054	0.0015

Within rows: means with different superscript differed significantly ($P < 0.05$).
Abbreviations: SEM – standard error of mean. Groups: Control, non injected; Nano-Ag, injected with silver nanoparticles; Glu, injected with glutamine; Nano-Ag/Glu, injected with silver nanoparticles and glutamine. FGF2, Fibroblast Growth Factor 2; VEGF, Vascular Endothelial Growth Factor; PAX7, Pair Box transcription Factor 7; PCNA, Proliferating Cell Nuclear Antigen; MyoD1, Myogenic Differentiation Factor; ATP1A1, ATPase.

the *VEGF* expression, there was a significant increase for the Nano-Ag (1.34) and also for Glu (2.08) treated chick embtros, which had the highest level of expression compared to the control group (0.57). When *Pax 7* was examined, the level of expression was lowest in the Nano-Ag group (0.91), however, differences between treatments were not significantly different. Nano-Ag/Glu (1.54) significantly increased the expression of *PCNA* compared to the other groups (Control: 0.92; Nano-Ag: 1.01; Glu: 1.07). Nano-Ag (1.57) and Nano-Ag/Glu (1.0) significantly increased the expression of *MyoD1,* compared to the other groups (Control: 0.68; Glu: 0.62). Furthermore, Nano-Ag (1.38) significantly increased the level of *ATP1A1* expression but only compared to the Controls (0.92) and the Nano-Ag/Glu group (0.80).

Discussion

The results of the present experiment show that colloids of silver nanoparticles administrated at a low level (50 ppm) do not negatively affect chicken embryo growth and development. The weight of embryos and organs, as well as biochemical indices, measured in the blood serum, did not point towards any negative changes, being in agreement with previous results from experiments carried out *in vitro*[28, 29], *in vivo* [30, 31, 32] and also *in ovo*[33, 34, 35]. However, the toxicity of silver nanoparticles remains controversial and is far from completely understood[36]. In the present study the indices for chicken embryo growth and development were also not affected by the administration of glutamine as well as glutamine conjugated with Nano-Ag. Glutamine is a natural component of the body, therefore, we supposed that when used at a low level it should be non-toxic, especially since glutamine has shown beneficial properties, preventing chemotherapy and radiation-induced toxicity in humans[37].

Nano-Ag, glutamine and the complex of Nano-Ag/Glu were non-toxic, however, the supplements were also not neutral in terms of the chicken embryo. Colloids of silver nanoparticles increased mRNA expression of genes *FGF2* and *VEGFA*, which suggests that silver nanoparticles may activate growth and development at the molecular level, as has indeed been demonstrated previously[11]. In the present experiment the expression of *MyoD1* and *ATP1A1* was also up-regulated by silver nanoparticles, when compared to the control group. The up-regulation of *MyoD1* indicates that nanoparticles of silver activate myogenesis. In general this process is strictly limited by oxygen and nutrient availability and consequently by the rate of metabolism, especially by the O_2 dependent mechanisms of oxidation[38,39] and the amount of energy stored within the cell. The energetic state of the cells is related to the activities of the protein, encoded by the *ATP1A1* gene, which belongs to the family of P-type cation transport ATPases - Na^+/K^{+}[40]. The activity of such ATPases is related to the balance of O_2 in the mitochondrial respiratory chain reactions[41]**.** Non-adequate amounts of O_2 in the muscle can limit its development. Silver has the ability to absorb O_2, and it has been suggested, that oxygen species coexist on the surface of silver[42, 43]. We

propose that silver nanoparticles, acting as small delivery vehicles are capable of crossing cell membranes and of transporting O_2 directly to muscle cells. In the present experiment Nano-Ag treatment insignificantly decreased O_2 consumption at the end of embryogenesis compared to the control group (Figure 4). This may indicate that the requirement for O_2 was partially covered by O_2 transported by Ag, however, further experiments are needed to confirm this tendency.

Silver nanoparticles, administered to the embryos increased the expression of FGF2 and VEGFA at the mRNA and protein levels. The *FGF2* gene is involved in the regulation of muscle growth as a strong stimulator of myoblast and satellite cells proliferation and an inhibitor of their differentiation[1]. The activities of *FGF2* during embryogenesis decrease around hatching when the number of muscle cells is almost stable [43]. However, in the present experiment FGF2 was up-regulated both at the mRNA and protein levels at day 20 (just before hatching). This may indicate that nanoparticles are capable of sustaining muscle cell proliferation and/or inhibiting signals that turn on the process of differentiation in these muscle cells. Furthermore, VGFA was up-regulated, indicating that nanoparticles may promote the growth of vascular endothelial cells and stimulate angiogenesis.

Silver nanoparticles increased the mRNA expression of *MyoD1*, which controls embryonic myogenesis at all stages of muscle development, as well as in adult tissue[44, 45, 46, 47, 48]. *MyoD1* is expressed in proliferating myoblasts and encodes for nuclear proteins, which regulate muscle cell differentiation by cell cycle arrest stimulation[39, 49]. Cell cycle regulation processes are dependent on PCNA activities, which was not affected by our Nano-Ag. We therefore expect that the activation of extra signals involved in proliferation of muscle's cells also requires an extra amount of energy and/or nutrients to finish the process of proliferation and to start the process of differentiation.

Interestingly, the results observed upon injection of Nano-Ag were contrary to the results observed after the application of silver nanoparticles conjugated with glutamine. The expression of *ATP1A1* at the mRNA level decreased compared to Nano-Ag, yet was similar to values for the control group. Furthermore, the expression of *VEGF* and *MyoD1* was found to be down-regulated, indicating contrary effects to those obtained with Ag-Nano. However, *MyoD1* was expressed at a lower level in the Nano-Ag/Glu group than in the Nano-Ag group, although still at a higher level than in the control group. The expression of *PCNA* increased in comparison to all the other groups. *PCNA* plays an important role in nucleic acid homeostasis, as a component of the replication of DNA synthesis and DNA repair[50].

Glutamine, after glucose, is the next primary carbon source for ATP production and DNA biosynthesis in proliferating mammalian cells[51]. We propose that glutamine, when transported with silver nanoparticles is protected against breakdown as well as facilitating the direct delivery of this compound to cells. When we observed results obtained by visualization of the samples, the average cell number per area was found to be lower in all the injected groups

compared with the control group, but the average number of nuclei in proportion to the number of cells was found to be higher. Furthermore, the fibre area was significantly increased in the treatment groups, compared to the control group. Thus in every case major effects were seen for the Nano-Ag and Nano-Ag/Glu groups compared with the controls. These results indicate a significant role for silver nanoparticles in muscle development, a role that is sustained or even enhanced by the use of a Nano-Ag/Glutamine complex. Moreover, the application of nano silver with glutamine significantly increased the mass of the pectoral muscle as compared with the control group, and also when compared with the Nano-Ag and Glu groups. This overall promoting effect of Nano-Ag/Glu on muscle development supports our hypothesis that silver nanoparticles act as carriers of glutamine to the cells. Indeed, provision of muscle cells with glutamine in this way would be expected to improve nucleic acid synthesis and metabolic programing within cell, increasing their fibre area and consequently muscle mass.

>> Nano-particles of silver may be useful as a potential carrier of nutrients and/or as an active agent

Conclusion

Nanoparticles of silver, glutamine and complexes of Nano-Ag with glutamine were without negative effects on embryo development. Nano-Ag and Nano-Ag/Glu affected the expression of genes responsible for muscle development during embryogenesis. However, the molecular effects were not consistent for Nano-Ag compared with those for Nano-Ag/Glu. Nano-Ag and Nano-Ag/Glu affected muscle morphology by increasing the number of nuclei per cell, and by increasing fiber area. Moreover, the complex of Nano-Ag with glutamine increased muscle mass.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

FS and AC participated in the design and supervised the experiment. FS drafted the manuscript. FS, LP and AH carried out all of the laboratory analyses and participated in the statistical analysis. PH participated in the design and supervised microscopy analyses. ES, MS and TN participated in the molecular studies and in drafting the manuscript. AC and ES participated in the design and coordination, and helped draft the manuscript. All authors read and approved the final manuscript.

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